ISOLATION OF BACTERIOPHAGES SPECIFIC TO *ESCHERICHIA COLI*, *STAPHYLOCOCCUS AUREUS* AND *PSEUDOMONAS AERUGINOSA* IN LIVESTOCK FROM WEST-CENTRAL TEXAS.

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ISOLATION OF BACTERIOPHAGES SPECIFIC TO *ESCHERICHIA COLI*, *STAPHYLOCOCCUS AUREUS* AND *PSEUDOMONAS AERUGINOSA* IN LIVESTOCK FROM WEST-CENTRAL TEXAS.

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ABSTRACT

Cross-species infections of methicillin-resistant *Staphylococcus aureus* (MRSA) from livestock to humans have been reported. Prior studies have isolated *S. aureus* and associated bacteriophages from dairy cattle. This research is undertaken to isolate bacteriophages specific for *Escherichia coli*, *S. aureus* and *Pseudomonas aeruginosa* from 13 beef cattle, 15 sheep and 12 goat fecal samples from a ranch in Tom Green Co., Texas. Phage enrichment was carried out using *E. coli* American Type Culture Collection (ATCC) strain 23848, *S. aureus* ATCC strain 13565 and *P. aeruginosa* ATCC strain PA01. Phage presence was detected using lawn spotting and reconfirmed using plaque assay. Bacteriophages specific to *E. coli*, *S. aureus* and *P. aeruginosa* were isolated from fecal samples of sheep and goats, while only *S. aureus* phages were isolated from cattle feces. Statistical analysis using Fisher's exact test and nested set modeling showed significant differences in phage isolation success between livestock types.

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INTRODUCTION

Background information. Bacteriophages, or 'eaters of bacteria,' were first discovered in 1915 by bacteriologist Frederick Twort, and a second time independently in 1917 by the microbiologist Felix d'Herelle. Bacteriophages refer to any virus that infects bacteria as host organisms (Douglas 1975, Weinbauer 2004).

Recently, the medical community has become very interested in bacteriophages for therapeutic purposes. The field of phage therapy – bacteriophages as antibacterial agents – has advanced considerably including a recent surge of interest in Western medicine as an alternative to antibiotics (Häusler 2006, Kwiatek 2012, Weinbauer 2004). The relative ease by which phages can be isolated and produced compared to antimicrobial or antibiotic agents and the increasing resistance of bacterial pathogens to traditional antibiotics both serve as important reasons for the advancement of phage therapy (Balogh et al. 2010, Gill and Hyman 2010, Kwiatek 2012, Shi et al. 2010). With the increasing utilization of bacteriophages in phage therapy, an understanding of the ecology of phages would be expected to contribute to the field of bacteriophage therapy by revealing the range of habitats occupied by phages medically useful for treating bacterial diseases of humans (Weinbauer 2004). Additionally, bacteriophages represent a more economical and environment-friendly alternative to the environmentally-damaging use of chemical bacteriocides in agricultural industries (Balogh et al. 2010). Finally, phages are also employed in water treatment techniques as highly effective indicator organisms for the presence of bacteria, being commonly useful when analyzing for possible contamination of potential reservoirs for pathogenic bacteria (Contreras-Coll et al.

2002, Douglas 1975, Kenard and Valentine 1974).

This review will examine various aspects of bacteriophage ecology, particularly focusing on phages specific to the bacterial species *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Of these three bacteria, *S. aureus* and *E. coli* are known to have virulent forms that are responsible for animal-to-human transmissions, either directly via contact with human tenders or through their produced foodstuffs (Beutin et al. 1993, Ferber 2010, Garcia et al. 2007, Garcia et al. 2009, Kwiatek et al. 2012, Lee 2003, van Loo et al. 2007, Shi et al. 2010). *S. aureus* and *P. aeruginosa* are known to cause mastitis in dairy cattle as well (Garcia et al. 2009, Kwiatek et al. 2012, Osborne et al. 1981, Shi et al. 2010). Mastitis is an infection of the mammary glands that can result in decreased milk yields, or may contaminate milk supplies with bacterial enterotoxins (Ikeda et al. 2005, Petersson-Wolfe et al. 2010).

***Staphylococcus aureus* and associated bacteriophages.** As previously mentioned, *S. aureus* has been identified as a common causative microorganism of mastitis in cattle (Garcia et al. 2009, Kwiatek et al. 2012, Shi et al. 2010). Epidemiologically, *S. aureus* are important as common colonizers and infectious agents of humans (Frank et al. 2010, Gleckman et al. 1988, Kluytmans et al. 1997). Infected individuals may be at risk for surface skin and soft tissue infections, endocarditis, osteomyelitis, meningitis, bacteremia, and pneumonia (Frank et al. 2010, Gleckman et al. 1988). Morphologically, *S. aureus* are gram-positive cocci-shaped bacteria (Gleckman et al. 1988). Chambers (2001) have noted similarities in the recent appearance of methicillin-resistant *S. aureus* (MRSA) with the spread of penicillin-resistant strains during the 1940s and 1950s. Notably, the development of both types of antibiotic resistant *S. aureus* were strongly correlated with clinical settings, attributable to the

prevalence of antibiotic use in such environments and that humans are a natural reservoir of *S. aureus*. However, the same author (Chambers 2001) notes that MRSA infections are not exclusive to clinical settings, and such resistant strains have also been acquired from other sources as well.

Research on MRSA has revealed evidence that cross-species infections of MRSA from livestock, such as pigs and cattle, to their human tenders are highly common, and a significant portion of MRSA diagnosed in human populations may have originated from non-human animal environments (Ferber 2010, van Loo et al. 2007). In addition to its epidemiological threat, *S. aureus*-associated mastitis incurs an economic cost on the dairy industry as the bacteria itself infects the udder and adversely affects milk production by reducing yield or contaminating the supply with dangerous enterotoxins (Ikeda et al. 2005, Petersson-Wolfe et al. 2010)

To date, a strong focus that exists is to isolate and identify *S. aureus* from dairy cattle as the bacteria commonly contaminate milk and dairy-based products (Garcia et al. 2007, Garcia et al. 2009, Kwiatek et al. 2012, Lee 2003, van Loo et al. 2007, Shi et al. 2010). In June 2000, over 10,000 cases of food poisoning were reported in Osaka, Japan. The cause of this was a malfunction in the temperature control systems of a plant that produced skimmed milk powder, allowing the proliferation of *S. aureus*. While the bacteria were killed by pasteurization, heat-resistant staphylococcal enterotoxins remained in the reconstituted milk which eventually reached the consumers (Ikeda et al. 2005).

Milk from dairy cattle appears to be the most common source of *S. aureus* and MRSA strains (Kwiatek et al. 2012, Lee 2003). In non-dairy livestock, successful isolation of *S.*

aureus has been reported in beef cattle, chicken and pigs, while MRSA were reported in chickens only (Lee 2003).

Between May 2001 and April 2003, Lee (2003) successfully isolated *S. aureus* from 421 out of 1,913 feces, milk, feed material, joint, trachea, uterus, and meat samples collected from 15 slaughterhouses, seven meat processing facilities, 58 feedlots, and 11 food stores located throughout South Korea. According to this study, *S. aureus* were successfully isolated from dairy and beef cattle, chickens and pigs. MRSA strains were identified via the presence of the *mecA* gene, testing positive in 12 dairy cattle and three chicken samples. No MRSA strains were detected in the beef cattle or the pigs.

In the investigation conducted by Lee (2003), 10 g of feces, feed and meat, or 10 ml of milk, or swabs of joint, trachea and uterus were inoculated in 100 ml of staphylococcus broth or Trypticase soy broth and incubated at 35°C for 20 h. The inocula were transferred to Baird-Parker agar and incubated again at 35°C for 24 to 48 h. *S. aureus* bacteria were identified using biochemical methods such as Gram staining, colonial morphology, coagulase and urease assays, and the API Staph Ident system (Lee 2003).

Bacteriophages ΦH5 and ΦA72, specific to *S. aureus*, have been successfully isolated from raw or bulk tank milk from dairy farms (Garcia et al. 2007, Garcia et al. 2009). Literature has revealed that the phages may also be found in processed dairy products such as cheese. In 2009, García et al. (2009), identified eight different phages, isolated from milk and cheese samples, each collected from 72 different dairy farms and 3 cheese factories in northern Spain. All 75 samples screened positive for phage presence, achieved by culturing onto mastitis milk-derived strains of *S. aureus* and incubated overnight at 37°C with agitation. Samples were subjected to centrifugation at 13,000 × *g* for 5 min followed by

filtration. The presence of phages was then assessed via plaque assays against various strains of *S. aureus*. All phages isolated in this study were temperate in nature. To demonstrate the temperate nature of the phages, García et al. (2009) isolated bacterial colonies, suspected to be resistant to infection, from lysis plaques. These isolated colonies were then exposed to mitomycin C to induce prophage release.

In addition to milk samples, other possible sources of bacteriophages specific to *S. aureus* may include sewage effluent, soil, and straw from cowsheds. In 2011, *S. aureus* phages were isolated from wastewater collected from a dairy farm in China. A three-step enrichment process using *S. aureus* grown in Luria-Bertani liquid medium yielded a lytic, tailed phage with morphology matching that of the order *Caudovirales*. The presence of such phages was confirmed by plaque assay (Li and Zhang 2011).

Two novel bacteriophages specific to *S. aureus* were reported to be isolated from sewage, soil and straw samples collected from cowsheds. Phages were screened by culturing onto bacterial hosts, concentrated by centrifugation at 8,000 rpm for 20 min and filtered using 0.45-μm filters, and finally confirmed via plaque assay using *S. aureus* as host (Yoon et al. 2010). It is likely that the phages originated from cows, which contaminated the soil, straw and other surfaces through defecation. These phages may eventually be washed into sewage holding tanks.

Most recently Kwiatek et al. (2012) isolated bacteriophages lytic against *S. aureus* from milk via standard enrichment using a mixed culture of three randomly selected bacterial strains; *S. aureus* American Type Culture Collection (ATCC) 43300, ATCC 25923, and *S. aureus* MRSA 643. Phage lysis was forcibly induced using chloroform, and centrifuged. Phage presence was detected by applying supernatant to bacterial lawn cultures. After a 24 h

incubation period, lytic zones were extracted, filtered and subjected to successive single-plaque isolations to produce pure phage cultures (Kwiatek et al. 2012).

***Pseudomonas aeruginosa* and associated bacteriophages.** *P. aeruginosa* is an aerobic gram negative, rod-shaped bacillus bacterium (Driscoll et al. 2007, Gaby and Hadley 1957). Similar to *S. aureus*, *P. aeruginosa* are also identified to be epidemiologically important human pathogens responsible for pneumonias, urinary tract infections (UTIs), bloodstream infections, and surface skin infections (Driscoll et al. 2007). Antimicrobial-resistant strains of *P. aeruginosa* are also highly common, with the reported emergence of strains resistant to antibacterial agents such imipenem, fluoroquinolones and cephalosporins, as well as multidrug-resistant variants (Driscoll et al. 2007). Additionally, *P. aeruginosa* have also been identified as causative microorganisms for mastitis in cattle (Osborne et al. 1981).

Compared to *S. aureus*, research literatures on *P. aeruginosa*-caused mastitis infections appear to be much less common (Osborne et al. 1981). This thesis represented an opportunity to expand on the understanding of the ecology of *P. aeruginosa* and associated bacteriophages in an animal environment.

In 1979, a herd of dairy cattle was involved in a mastitis outbreak. During an investigation, *P. aeruginosa* was isolated from milk and udder tissue. It was suspected that bacterial infection was spread via a contamination that was spread during the intravenous administration of antibiotics (Osborne et al. 1981).

One study performed genomic analysis of dairy-based bacteriophages within the family *Siphoviridae* (phages with long, non-contractile tails) in the order *Caudovirales* and identified *Staphylococcus* phage PVL and *Pseudomonas* phage D3 as dairy phages. The two

phages were identified to be specific to the bacterial species *S. aureus* and *P. aeruginosa* respectively (Brüssow and Desiere 2001).

In addition to *S. aureus*, it is possible to isolate *P. aeruginosa* phages from cow feces and secretions from cows, such as uterine discharges. According to Santos et al. (2011), *P. aeruginosa* bacteriophages P2S2 and P5U5 have been isolated from manure and uterine secretions of dairy cows. Samples were centrifuged at 3000 × *g* for 25 min at 4°C, and then ultrafiltered using membranes with 0.22 μm pore sizes.

***Escherichia coli* and associated bacteriophages.** In addition, this study will also attempt to isolate *E. coli* and associated bacteriophages from non-dairy livestock using the same samples. This would serve as an expected positive control to which the results of *S. aureus* and *P. aeruginosa* could be compared, as *E. coli* and *E. coli*-specific phages are found in beef cattle, sheep and goats (Beutin et al. 1993, Calci et al. 1998, Kudva et al. 1998, Kudva et al. 1999, Niu et al. 2009, Oot et al. 2007, Valcour et al. 2002). *E. coli* are gram-negative, facultative anaerobic bacilli that are generally associated with digestive microflora (Gleckman et al. 1988, Duval-Iflah et al. 1981). However, Shiga toxin-producing *E. coli* (STEC) variants are associated with a spectrum of illnesses including diarrhea, hemolytic uremic syndrome, and acute renal failure (Valcour et al. 2002). The most important of these STEC strains is O157:H7, named for the serotype of the two antigens which are used in identification tests of *E. coli* strains (Beutin et al. 1993). Unlike the mastitis-causing *S. aureus*, which constantly threatens the dairy industry via the contamination of milk, the *E. coli* O157:H7 strain affects the beef industry by transmitting to meat products destined for human consumption (Oot et al. 2009).

Cattle have been identified as a natural reservoir for STEC (Niu et al. 2007, Oot et al. 2009, Valcour et al. 2002). Human STEC infections have also been closely linked to the density of beef cattle and associated manure in rural areas of Ontario, Canada, in an investigation by Valcour et al. (2002). No association could be found between human infections, and sheep and goat density. This was suspected to be due to sheep and goat densities being too low. However, other research has established that sheep and goats are important reservoirs for STEC as well (Beutin et al. 1993, Kudva et al. 1998).

A study in 1998 by Calci et al. (1998) compared the prevalence of bacteriophages lytic to *E. coli* in fecal samples from different animal species. The study reported positive phage isolation in beef cattle, sheep and goats, although it was noted that sheep and goats had the lowest mean density of bacteriophage, based on the number of plaque-forming units per gram of feces.

In 2007, Oot et al. (2007) isolated *E. coli* O157:H7 and associated bacteriophages from fecal samples collected from cattle at a commercial feedlot. One gram of fecal samples was homogenized in 9 ml of phosphate buffer solution (PBS). Aliquots of 300 µl of supernatant were inoculated with 1.2 to 1.5 ml of log phase *E. coli* O157:H7 culture and incubated overnight at 37°C with agitation. The enrichment culture was centrifuged at 3000 × *g* for 15 min, and the collected supernatant was then spot tested for phage presence. *E. coli* O157 bacteria screening was achieved by enrichment and isolated via O157-selective CHROMagar growth media. Serotypes were then determined by Enzyme Linked Immunosorbent Assay (ELISA) tests. *E. coli*-specific bacteriophages were isolated via enrichment in ~97% of fecal samples but the bacteria were only isolated in ~27% of the samples. This led to the conclusion that a negative correlation between bacteria and phage population which Oot et al.

(2007) suggested was caused by resident phages preventing colonization of ruminant guts by *E. coli* O157:H7.

More recently in 2009, Niu et al. (2009) had successfully isolated bacteriophages specific to *E. coli* O157:H7 from cattle at another commercial feedlot. Similar to Oot et al. (2007), Niu et al. (2009) attempted to observe correlations between bacteria and phage prevalence while including various temporal and environmental variations. Sources of phages were drinking water from water troughs, dry fecal pats from pen floors, fecal samples from cattle rectum, and fecal slurry from pen floors. Fecal slurry was only available when poor drainage conditions persist. Fecal samples were homogenized in diluent, and filtrate samples were centrifuged at 11,000 × *g* for 10 min, and filtered through a 0.22-µm syringe filter. Aliquots of filtrate from pats and rectal feces, or centrifuged slurry filtrate, or concentrated trough water were screened for phages. Phage screening was achieved by inoculating 450 µl of processed samples with 50 µl of early log phase *E. coli* and incubated for 1 h at 37°C. Spot platting were used to observe for phage activity via the appearance or absence of viral plaques. In parallel to phage isolation, Niu et al. (2009) also carried out bacteria isolation of *E. coli*. One gram of fecal samples were enriched in 9 ml of EC broth and incubated at 37°C for 6 h. Immunomagnetic separation followed, by incubating 1 ml of the enrichment culture with 20 µl of anti-*E. coli* 0157 magnetic beads for 30 min. The magnetic beads were washed in PBS thrice and the collected bacterial suspension was then plated onto sorbitol MacConkey agar. Further differentiation was achieved by isolating non-sorbitol-fermenting colonies and testing for the presence of the O157 antigens by agglutination with an *E. coli* O157-selective latex kit.

Niu et al. (2009) noted a significantly greater prevalence of phages in manure slurry compared to fecal pats and rectal feces, achieving successful phage isolation in 94.6%, or 35 out of 37 slurry samples compared to just 26.5% (109 out of 411) of fecal pat samples and 23.8% (76 out of 320) of rectal fecal samples. In contrast, drinking water sources yielded *E. coli*-specific phages in 21.8%, or 19 out of 87 water trough samples. Niu et al. (2009) suggest that phage prevalence is positively correlated with moisture, and shedding of phages and bacteria by cattle might also dictate the probability of successful phage and bacteria isolation. Comparison of the results of phage and bacteria isolation in rectal fecal samples revealed that 16.9% of samples were positive for *E. coli* O157:H7-specific phage presence but negative for host bacteria. Only 6.9% were positive for both phage and bacteria. Lastly, the study by Niu et al. (2009) demonstrated a negative correlation between *E. coli* O157:H7 and phage prevalence, thus suggesting the bacteriophage activity reduces the bacteria population. This stands in contrast to the statistically-upheld correlations noted in studies on the use of coliphages as indicator organisms for coliform bacteria such as *E. coli* (Contreras-Coll et al. 2002, Kenard and Valentine 1974)

In 1989, Klieve et al. (1989) achieved bacteriophage induction of ruminal bacteria from sheep using mitomycin C. This yielded long, filamentous phage-like particles (PLP) matching that of pyocin 28, a bacteriocin produced by *P. aeruginosa* (Klieve et al. 1989). While this study did not analyze the nucleic acids of the filamentous particles, and therefore could not confirm the particles to be pyocin 28, Santos et al. (2011) demonstrated that *P. aeruginosa*-specific phages can exist in intestinal tracts of livestock.

Bacteriophage and host ecology in livestock. Given the economic importance of mastitis in dairy industry, most related research is focused on dairy livestock. The primary

sources of mastitis-associated *S. aureus* and *P. aeruginosa* bacteria are dairy livestock and their environment (Brüssow and Desiere 2001, Ferber 2010, Garcia et al. 2007, Klieve et al. 1989, Lee 2003, Osborne et al.1981, Weinbauer 2004). Similarly, dairy cows and their associated environments also appear to be the primary source of bacteriophage specific to mastitis-associated *S. aureus* and *P. aeruginosa* (Brüssow and Desiere 2001, Garcia et al. 2007, Garcia et al. 2009, Kwiatek 2012, Li and Zhang 2011, Santos et al. 2011, Yoon et al. 2010). It should be noted that dairy animals are not the only source of mastitis, as *S. aureus* has been isolated from non-dairy livestock such as beef cattle, chickens and pigs (Ferber 2010, Lee 2003, Valcour et al. 2002).

The impetus for research into bacteriophages associated with such bacteria can be attributed to the increasingly common view of their use as effective biocontrol agents in the dairy industry (Balogh et al. 2010, Garcia et al. 2007, Garcia et al. 2009, Kwiatek 2012, Weinbauer 2004).

Overall, bacteriophages specific to *E. coli*, *S. aureus* and *P. aeruginosa* have been isolated from dairy cattle (Brüssow and Desiere 2001, Calci et al. 1998, Garcia et al. 2007, Garcia et al. 2009, Kwiatek 2012, Li and Zhang 2011, Yoon et al. 2010, Santos et al. 2011). In non-dairy livestock, *E. coli*-specific phages are expected to be isolatable from beef cattle, sheep and goats, which this survey expects to be utilizable as a control to which the isolation of phages specific to *S. aureus* and *P. aeruginosa* can be compared (Calci et al. 1998, Niu et al. 2009, Oot et al. 2007). In contrast, *P. aeruginosa*-specific phages have been reported to be successfully isolated from sheep (Klieve et al. 1989). *S. aureus*-specific phages have not been isolated from beef cattle, sheep and goats.

Possible sources for isolation of bacterial species *S. aureus* and *P. aeruginosa* are milk (Ikeda et al. 2005, Kwiatek et al. 2012, Lee 2003, Osborne et al. 1981, Petersson-Wolfe et al. 2010, Shi et al. 2010), and feces (Lee 2003, Valcour et al. 2002). In studies involving dairy livestock, milk and udders were primary sources for samples from which phages were isolated (Brüssow and Desiere 2001, Garcia et al. 2007, Garcia et al. 2009, Kwiatek et al. 2012, Osborne et al. 1981, Shi et al. 2010). Alternate sources from which phages could be isolated include sewage or wastewater (Calci et al. 1998, Li and Zhang 2011, Yoon et al. 2010), and feces (Calci et al. 1998, Niu et al. 2009, Oot et al. 2007, Santos et al. 2011). This literature review notes that *P. aeruginosa*-specific phages have not been reported to be isolated from drinking water yet.

The need to understand the microbial ecology of bacteriophages is an inevitable prerequisite to the successful isolation and acquisition of phages specific against the target bacteria. The relative simplicity by which phages can be isolated from the environment can be attributed to the fact that the natural environment of the bacteria of interest is likely to contain the specific phages capable of infecting and lysing the microorganism (Gill and Hyman 2010).

RESEARCH OBJECTIVES

This thesis attempted to isolate bacterial species *E. coli*, *S. aureus* and *P. aeruginosa*, and associated bacteriophages from environmental samples associated with beef cattle, sheep and goats. This investigation into non-dairy livestock excluded milk from sample collection. Instead this study focused on the collection of fecal and drinking water samples from which phage and bacteria isolation will be attempted.

All three bacterial species are potential human pathogens and therefore physical contact with livestock might lead to cross-contamination. It can be expected that the presence of bacteriophages is dependent on the presence of resident host bacteria. Successful isolation of phages specific to these bacterial species could serve as indicators of the presence of their respective bacterial hosts, and thus serve as an alternative to biochemical testing for potential bacterial pathogens. This thesis will use statistical analysis to find correlations in the prevalence of *E. coli*, *S. aureus* and *P. aeruginosa* bacteria and their associated bacteriophages.

MATERIALS AND METHODS

Study site. All environmental samples were collected from the Management, Instruction and Research (MIR) Center, Angelo State University. The MIR is a 6,000-acre ranch habitat in Tom Green Co., Texas that includes beef cattle, sheep, and goats that form the basis of this research.

The MIR Center usually houses all three livestock types in segregated holding stalls in a centralized location. Holding stalls dedicated to sheep, and goats were shaded while beef cattle were usually kept in larger, open-air stalls. However, it should be noted that beef cattle were relocated onto grazing pastures as part of their summer and fall rotation and were not present in their holding stores during the collection of all water samples, and fecal samples A to AN, which were used in bacteriophage isolation and the first segment of bacteria isolation. A second set of fecal samples AO to AZ, in the second segment of bacteria isolation, was collected after the beef cattle were returned to their holding stalls.

The ranch lacks a sewage disposal system. Instead, wastewater in the holding stalls is removed via direct evaporation from soil. Hence, alternate to collecting wastewater sample, this study will collect water samples from water troughs instead (Niu et al. 2009).

Collection of environmental samples. Thirteen water samples were collected from various water troughs from 23 July 2012 to 21 September 2012. Four fluid ounces or approximately 120 ml of water were collected from each water trough. On 23 July 2012, three water samples were collected, the first which was not in use to serve as a control, and two from fenced areas housing sheep. On 31 July 2012, another three water samples were collected, one being used by sheep, the second being used by goats and the third from another unused water trough as a second control. On 6 August, two water samples were

collected from water troughs in holding stalls utilized by sheep and three samples from water troughs in holding stalls used by goats. The last water sample collection was made on 21 September 2012, where two samples were collected from water troughs in fenced pastures housing beef cattle (Table 1). Collection of water samples after 21 September 2012 ceased as phage isolation results showed low success in isolating bacteriophages from water troughs. Instead, the focus of study shifted to fecal samples only.

TABLE 1. Water sample collection log.

Water Trough	Associated Livestock	Date	Time	Weather	Temperature	Additional Notes
A	Not in use	7/23/2012	3.45 PM	Slightly Cloudy	93°F/34°C	Not in use
B	Sheep		3.15 PM			-
C	Sheep		3.20 PM			-
D	Goat	7/31/2012	2.24 PM	Sunny (light rain earlier)	77°F/25°C	-
E	Not in use		2.26 PM			Not in use
F	Sheep		2.31 PM			-
G	Sheep	8/6/2012	3.34 PM	Sunny	99°F/37°C	-
H	Sheep		3.37 PM			-
I	Goat		3.46 PM			Shaded
J	Goat		3.49 PM			Shaded
K	Goat		3.52 PM			Shaded
L	Cattle	9/21/2012	4.32 PM	Sunny	93°F/34°C	-
M	Cattle		4.38 PM			-

It was noted that the appearance of feces from cattle, sheep and goats were different. To aid in differentiating feces from the different livestock types, collection of fecal samples did not begin until recently-vacated feces from cattle, sheep and goats were observed and noted for distinctions in physical appearance. This was important as sheep and goats were housed adjacent to each other, and the grazing pastures accommodating the beef cattle were also inhabited by donkeys.

Thirteen cattle, 15 sheep and 12 goat fecal samples were collected from 13 July 2012 to 26 October 2012. Approximately a 2 fluid oz. equivalent in volume of fecal material was collected in each sampling. On 13 and 20 July 2012, five and seven goat fecal samples were respectively collected from 12 randomly selected holding stalls out of a total of 13 holding stalls. Double-sampling was avoided by not repeating sample collection from the same stalls. Five sheep fecal samples were collected on 14 September 2012, each from different holding stalls and 10 more samples were collected on 26 October 2012 from the same five stalls. Three cattle fecal samples were collected from fenced pastures where grazing activities were occurring on 21 September 2012. Another 10 samples were collected from the same pastures on 5 October 2012. Finally, on 20 January 2013, four goat and four sheep fecal samples were collected from holding stalls. Four fecal samples were also collected from beef cattle, which were in holding stalls at the time of collection (Table 2).

TABLE 2. Fecal sample collection log.

Fecal	Associated Livestock	Date	Time	Weather	Temperature	Additional Notes
A	Goat	7/13/2012	9.30 PM	Cloudy/Light Rain	81°F/27°C	Shaded
B	Goat		9.32 PM			Shaded
C	Goat		9.34 PM			Shaded
D	Goat		9.35 PM			Shaded
E	Goat		9.38 PM			Shaded
F	Goat	7/20/2012	9.30 PM	Sunny	91°F/33°C	Shaded
G	Goat		9.32 PM			Shaded
H	Goat		9.35 PM			Shaded
I	Goat		9.37 PM			Shaded
J	Goat		9.39 PM			Shaded
K	Goat		9.45 PM			Shaded
L	Goat		9.47 PM			Shaded
M	Sheep	9/14/2012	3.02 PM	Cloudy	64°F/18°C	Shaded
N	Sheep		3.04 PM			Shaded
O	Sheep		3.06 PM			Shaded
P	Sheep		3.08 PM			Shaded

Q	Sheep		3.44 PM			Shaded
R	Cattle	9/21/2012	4.33 PM	Sunny	93°F/34°C	In grazing pastures
S	Cattle		4.34 PM			In grazing pastures
T	Cattle		4.40 PM			In grazing pastures
U	Cattle	10/5/2012	3.40 PM	Sunny (light rain earlier)	77°F/25°C	In grazing pastures
V	Cattle		3.43 PM			In grazing pastures
W	Cattle		3.45 PM			In grazing pastures
X	Cattle		3.50 PM			In grazing pastures
Y	Cattle		4.22 PM			In grazing pastures
Z	Cattle		4.25 PM			In grazing pastures
AA	Cattle		4.32 PM			In grazing pastures
AB	Cattle		4.33 PM			In grazing pastures
AC	Cattle		4.34 PM			In grazing pastures
AD	Cattle		4.35 PM			In grazing pastures
AE	Sheep	10/26/2012	2.17 PM	Cloudy	57°F/14°C	-
AF	Sheep		2.17 PM			-
AG	Sheep		2.19 PM			-
AH	Sheep		2.20 PM			-
AI	Sheep		2.29 PM			-
AJ	Sheep		2.29 PM			-
AK	Sheep		2.32 PM			-
AL	Sheep		2.34 PM			-
AM	Sheep		2.36 PM			-
AN	Sheep		2.39 PM			-
AO	Goat	1/20/2013	9.50 PM	Sunny	30°F/-1°C	-
AP	Goat		9.51 PM			-
AQ	Goat		9.53 PM			-
AR	Goat		9.59 PM			-
AS	Sheep		10.14 PM			-
AT	Sheep		10.16 PM			-
AU	Sheep		10.17 PM			-

AV	Sheep	10.21 PM	-
AW	Cattle	10.26 PM	In holding stalls
AX	Cattle	10.28 PM	In holding stalls
AY	Cattle	10.29 PM	In holding stalls
AZ	Cattle	10.30 PM	In holding stalls

Prior to each sample collection, field aseptic techniques were followed by washing hands and elbows with commercially-available bacteriological sanitizer. Disposable gloves were used to handle samples and replaced after each individual collection. Water and fecal samples were collected in sterile specimen containers and stored in ice for transportation. A total of 65 samples were collected for the purpose of this study.

Host bacterial cultures. Log phase cultures of *E. coli* ATCC 23848, *S. aureus* ATCC 13565 and *P. aeruginosa* ATCC PA01 were prepared in tryptic soy broth (TSB). Bacterial cultures were incubated at 35°C and turbidity was measured using a spectrophotometer (Coleman, Cincinnati, Ohio). Mid-log phase growth was achieved when optical density of 550 nm (OD_{550}) reached 0.8 absorbance levels (Kreuzer-Martin et al. 2005, Price and Raivio 2009).

Processing samples. Water and fecal samples are first processed based on recommended protocols for use in the Center for Phage Technology at Texas A&M University (2011). Water samples are filtered using 0.22 μm pore-sized membranes (Corning, Corning, N.Y.) without need for homogenizing into solution form. Fecal samples are homogenized using 0.85% physiological saline solution for at least 1 h with manual agitation every 20 min. The homogenate was then subjected to ultrafiltration using 0.22 μm pore-sized membranes (Texas A&M University 2011).

Micro-centrifuges were unavailable, or were of inappropriate size or speed for the purpose of this study. However, it is expected that sterile-filtration would be sufficient to separate phages in the supernatant from unwanted bacteria and detritus (Texas A&M University 2011). Due to the greater quantity of particulates within the supernatant, ultra-filtration took a greater amount of time than is typical. The quantity of 0.22 µm pore-sized filters available is the most limiting resource available to this study. This in turn limits the amount of data collection that this study could attempt.

Phage enrichment. This study followed a modification of the afore-mentioned protocol for phage enrichment (Texas A&M University 2011). Five milliliters of filtered homogenate was added to 40 ml of tryptic soy broth and inoculated with 2 ml of log phase bacterial cultures of either *E. coli*, *S. aureus* or *P. aeruginosa*. Enrichment cultures were incubated at 35°C for 36-48 hours with manual agitation every 6-8 hours, before being tested for the presence of bacteriophages.

Phage isolation. Aliquots of 0.3 ml of enrichment cultures were applied onto lawn cultures of respective bacteria on phage agar (Balogh et al. 2010). After a 36-48 h incubation period at 35°C, lawn spot plates were observed for the presence of cleared area indicating lytic phage activity. Plates displaying positive presence of phage activity were reconfirmed using double-layer plaque assay modified from the phage titration technique described by Brown and Connely (2009; p177-179).

Lytic zones from lawn spots were extracted using autoclaved toothpicks and homogenized in 1 ml of tryptic soy broth with agitation via vortex mixer. All 1 ml of homogenates were pipetted into 4 ml of soft phage agar, inoculated with 0.3 ml of log phase bacterial culture, and then plated onto prepared solid phage agar plates. After 24 h incubation

at 35°C, plaque assay plates were observed for the presence of viral plaques. This study considers positive phage isolation to be achieved if at least one clear viral plaque is observed. Occasionally, the plaque assays produced "clear" plates without observable bacteria, which were attributable to the quantity of viral particles being too high. This was resolved by repeating the extraction of viral plaques from lawn spots, and homogenizing in serial dilutions of 10^{-1}, 10^{-2}, and 10^{-3}. Repeated attempts have shown that homogenizing in 9 or 10 ml of tryptic soy broth, agitation by vortex for 20 s, and using 1 ml of the homogenate was sufficient to produce plaque assays. This reduced plaque-forming unit (PFU) counts enough to allow accurate observation of viral plaques.

Phage quantification/titration. This study elected to carry out phage quantification via titration on all water samples and goat fecal samples A-L which yielded positive phage isolation. Phage titration was achieved utilizing the methodology as described by Brown and Connely (2009). Serial dilutions of 10^{-1} and 10^{-2} were generated by adding 1 ml of water samples to 9 ml of tryptic soy broth, and then transferring the resultant solution to another 9 ml of tryptic soy broth. 1 ml of water samples or the serial dilutions were pipetted into 4 ml of soft phage agar, inoculated with 0.3 ml of log phase bacterial culture, and then plated onto prepared solid phage agar plates. After a 24 h incubation period at 35°C, plaque assay plates were observed for the presence of viral plaques, which were counted to calculate the quantity of PFU per ml in the original water sample, or PFU per cm^3 in the fecal sample.

Bacteria isolation. Retentate from filters used in processing 15 sheep fecal samples M-Q and AE-AN, and 13 beef cattle samples, R-AD, were tested for the presence of *E. coli*, *S. aureus* and *P. aeruginosa*. It was decided that filters used for processing goat samples would not be tested, as the filters had been kept in storage for up to approximately four months, and

thus there may have been significant loss in viability. Filters used to process fecal samples for sheep and beef cattle were stored for a shorter two to three months span. Retentate from filters were quadrant streaked onto MacConkey (Acumedia, Lansing, Mich.), Mannitol salt (BD) and Cetrimide agar (Acumedia), which were selective for *E. coli*, *S. aureus* and *P. aeruginosa* respectively.

To contrast the results from filter retentate, bacteria isolation was also carried out on processed homogenate from freshly-collected fecal samples. Four sheep fecal samples, AO-AR, four goat samples, AS-AV, and four beef cattle samples, AW-AZ, were collected and homogenized on the same day in 0.85% physiological saline solution for the purpose of bacterial isolation. Homogenates were similarly quadrant streaked to selective media using autoclaved cotton swabs.

Suspect colonies were visually compared to the colony morphology of control cultures of *E. coli*, *S. aureus* and *P. aeruginosa* grown on their respective selective media. Two suspect colonies from each medium were then extracted and further differentiated using Gram staining.

Gram staining followed the protocol recommended by Brown and Connely (2009; p109-111). Suspected bacterial colonies were collected from selective growth media and spot-inoculated on tryptic soy agar (TSA) (Acumedia) plates and incubated for 24 h at 35°C. Bacterial cultures were applied and heat-fixed onto microscopic glass slides. Bacteria were stained in crystal violet for 20 s and washed with distilled water. This was followed by staining with Gram's iodine for 1 min before being decolorized with ethanol and washed with distilled water. Counterstaining was achieved using Safranin where bacteria were

stained for 20 s before washing with distilled water. Slides were blotted dry with bibulous paper and observed under oil immersion microscopy.

To characterize *E. coli*, Gram-negative rod-shaped bacteria selectively isolated on MacConkey agar were differentiated using the indole test (MacWilliams 2012). Suspected bacterial colonies displaying red coloration positive for lactose fermentation were extracted and inoculated in tryptone broth and incubated at 35°C for 48 h. Cultures were then tested using Kovac's reagent for the presence of indole caused by metabolic degradation of tryptophan.

To characterize *S. aureus*, bacterial colonies displaying yellow coloration due to mannitol fermentation are further characterized via Gram stain. Gram-positive cocci-shaped bacteria colonies isolated on Mannitol salt ager were further differentiated using a latex agglutination test (Hardy, Santa Maria, Calif.) for the presence of coagulase clumping factor and protein A associated with *S. aureus* based on protocols by Brown and Connely (2009; p479-480).

To characterize *P. aeruginosa* colonies isolated on Cetrimide agar, visually suspicious bacterial colonies with yellow-green pigmentation were examined (Acumedia 2011). Gram-negative rod-shaped bacteria were further characterized by using an oxidase test (BD, Sparks, Md.). The oxidase test allows determination of the presence of cytochrome oxidase which exists in high concentrations in *P. aeruginosa* (Gaby and Hadley 1957). Bacteria testing positive for oxidase are then inoculated in asparagine broth and incubated at 35°C for 48 h. Bacteria cultured in asparagine broth are then tested for blue fluorescence under ultraviolet light (Kominos 1972, Price and Ahearn 1988).

It should be noted that this study can only presumptively identify *E. coli*, *S. aureus* and *P. aeruginosa* using the bacterial identification tests. For example, the latex agglutination test

for *S. aureus* has a 97% reliability correlation (Brown and Connely 2009). Hence, positive identifications via the methodologies described are not a complete determination, and results should be viewed only as a high probability of being the respective bacteria (Gaby and Hadley 1957, Heizmann et al. 1988).

The lack of successful isolation of *S. aureus* bacteria in both the retentate and homogenate led to an attempt to isolate bacteria by enrichment. Aliquots of 10 µl of the homogenates were added to 20 ml of TSB with 7.5% salt concentration. Enrichment cultures were incubated overnight. Quadrant streaks were made on MSA using the enriched cultures. Three isolated colonies were then selected based on similar morphology to a control streak of *S. aureus* and subjected to Gram staining. Gram-positive cocci are then further tested using *S. aureus*-specific latex agglutination tests.

Statistical analysis. Statistical analysis focuses on data from fecal samples. Data was inputted onto an Excel Datasheet as a contingency table and analyzed using R statistical software. Fisher's exact test was determined to be the most appropriate analysis given the small sample sizes available to this study (Agresti 1992). Firstly, Fisher's exact test was used to test for independence in paired comparisons of isolation success-failure ratios of *E. coli*, *S. aureus* and *P. aeruginosa*-specific phages between livestock types. Independence was also tested in paired comparisons of isolation success-failure ratios of *E. coli*, *S. aureus* and *P. aeruginosa*-specific phages within each livestock type.

To observe the interactions between variables in bacteriophage isolation, a nested set model with mixed-effects to calculate the chi-squared *p*-value was used. Livestock types and bacteria-specific phage types were considered as fixed effect variables. As each sample was tested thrice for each bacteria type, samples were grouped. Given the unknowable quality of

the samples, this variable was considered to be random, and utilized in the analysis as a random-effect variable. The interactions between each explanatory variable were compared using the lowest Aikake information criterion (AIC).

Results from bacterial isolation were also analyzed using a nested set model with mixed-effects to calculate the chi-squared *p*-value. Correlations were sought between bacteria isolation success with bacteria type, livestock type, and sampling type. The sampling type dictated if the sample were taken from filter retentate and homogenate. Once again, the best-explained interaction between explanatory variables was sought by observing for the lowest AIC. As no goat-associated retentate was available for bacteria isolation, that subset of samples was excluded from analysis. Instead, only information collected from bacteria isolation from sheep and beef cattle samples were analyzed.

RESULTS

Results for phage isolation. Bacteriophages specific to *E. coli* have been isolated from the control troughs and water troughs utilized by goats and cattle. *P. aeruginosa*-specific phages were isolated from water troughs utilized by goats only. No phages specific to *S. aureus* were successfully isolated from water troughs. The probability of isolating *E. coli* and *P. aeruginosa* from fecal material from all three livestock types are calculated to be 18.2% and 9.1% respectively by averaging phage isolation success over total sample size (Table 3). As previously mentioned, early comparison of isolation successes between water and fecal samples showed that the latter gave greater number of positive isolations. This thesis noted the possibility that increasing the sample sizes may eventually lead to successful phage isolation of *S. aureus*-specific phages from water samples, or isolation of any type of phage from sheep via drinking water.

TABLE 3. Phage isolation success rates and averaged probability for water sample.

	Successful phage isolation									
	control		goat		sheep		cattle		Total	Probability
E. coli	1	50.0%	1	25.0%	0	0.0%	1	50.0%	2	18.2%
S. aureus	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%
P. aeruginosa	0	0.0%	1	25.0%	0	0.0%	0	0.0%	1	9.1%
No. of Samples	2		4		5		2		11	

Bacteriophages specific to *E. coli*, *S. aureus* and *P. aeruginosa* were isolated from fecal samples of sheep and goats, while only *S. aureus* phages were isolated from cattle samples (Table 4). The probability of isolating *E. coli*, *S. aureus* and *P. aeruginosa* from fecal material across all three livestock types are calculated to be 27.5%, 45.0% and 10.0% respectively.

TABLE 4. Phage isolation success rates and averaged probability for fecal sample.

	Successful phage isolation							
	goat		sheep		cattle		Total	Probability
E. coli	4	33.3%	7	46.7%	0	0.0%	11	27.5%
S. aureus	3	25.0%	7	46.7%	8	61.5%	18	45.0%
P. aeruginosa	3	25.0%	1	6.7%	0	0.0%	4	10.0%
No. of Samples	12		15		13		40	

Results for phage titration. The natural bacteriophage levels in all water samples appear too low to produce a quantifiable titer even after using undiluted samples. In contrast, *E. coli*-specific phage levels in fecal sample A and C were titrated at approximately 6,500 PFU/cm^3 and 42,000 PFU/cm^3 respectively. Fecal samples which yielded positive phage presence but did not have sufficient natural phage levels to achieve successful quantification include *E. coli*-specific phages in fecal samples K and L, *S. aureus*-specific phages in fecal samples C, D, K and L, and *P. aeruginosa*-specific phages in fecal sample F and K.

Fecal A: 13,000 PFU/ml * $\frac{120ml}{\sim 60cm3}$ = ~26,000 PFU/cm^3

Fecal C: 84,000 PFU/ml * $\frac{120ml}{\sim 60cm3}$ = ~168,000 PFU/cm^3

Results for bacterial isolation. Bacteria isolation of filter retentate from twenty-eight sheep and beef cattle fecal samples showed that *E. coli* had the highest prevalence with a successful isolation rate of 39.3% from the tested samples, identified using Gram staining and testing for indole production. 14.3% of the samples yielded positive isolation of *P. aeruginosa*, identified using Gram staining, cytochrome oxidase presence and UV fluorescence. This study was unable to isolate *S. aureus* bacteria via Gram stains and latex agglutination tests from any of the sheep and beef cattle samples (Table 5).

TABLE 5. Bacteria isolation using filter retentate as sample type.

	Successful bacteria isolation from retentate							
Bacteria	**Goats**		**Sheep**		**Cattle**		**Total**	
E. coli	-	-	8	53.3%	3	23.1%	11	39.3%
S. aureus	-	-	0	0.0%	0	0.0%	0	0.0%
P. aeruginosa	-	-	1	6.7%	3	23.1%	4	14.3%
No. of Samples	0		15		13		28	

In contrast, bacterial isolation on homogenate from twelve newly-collected sheep, goats and beef cattle fecal samples isolated *E. coli* bacteria from all of the tested homogenates. In contrast, this study was unable to isolate *S. aureus* and *P. aeruginosa* from any of the tested homogenates (Table 6). All bacterial enrichment for *S. aureus* on homogenate samples also resulted in negative successful isolation and identification.

TABLE 6. Bacteria isolation using freshly-collected homogenate as sample type.

	Successful bacteria isolation from homogenate							
Bacteria	**Goats**		**Sheep**		**Cattle**		**Total**	
E. coli	4	100.0%	4	100.0%	4	100.0%	12	100.0%
S. aureus	0	0.0%	0	0.0%	0	0.0%	0	0.0%
P. aeruginosa	0	0.0%	0	0.0%	0	0.0%	0	0.0%
No. of Samples	4		4		4		12	

To compare the distribution of *E. coli* and associated phages among the 28 sheep and beef cattle samples, 14.3% (4/28) of samples produced positive isolation of both bacteria and phage, 25.0% (7/28) of the samples produced just the bacteria while 10.7% (3/28) had bacteriophages only. *P. aeuginosa* bacteria were successfully isolated from 14.3% (4/28) of sheep and beef cattle samples without phages, while 3.57% (1/28) yielded *P. aeuginosa*-specific bacteriophages only. No samples yielded both *P. aeruginosa* and associated

bacteriophages. As a comparison, *S. aureus*-specific phages were isolated from 53.6% (15/28) of sheep and beef cattle samples but no host bacteria were successfully isolated.

Taking into account that beef cattle and sheep fecal samples M-AN were tested thrice for each respective type of bacteria and phage, the findings of this study showed that 22.6% (19/84) of the tested samples produced bacteriophages only, while 13.1% (11/84) of the tested samples resulted in bacteria only. Only 4.76% (4/84) of the tested samples had successful isolation of both the bacteria and phage. The low association in the results of bacteriophage and bacteria isolation is indicative that a negative correlation existed between natural phage and bacteria populations.

Results for statistical analysis. The expected probabilities of isolation of *E. coli*, *S. aureus* and *P. aeruginosa*-specific phages were 0.275, 0.450, and 0.100 respectively (Table 4). The expected probabilities were calculated as the averaged isolation successes of the respective bacteriophage across all three livestock types.

Testing for independence of phage isolation probability between livestock types, Fisher's exact test observed no significant differences in isolation rates of phages specific to all three bacterial species between sheep and goat. Significance was observed when comparing isolation rates of *E. coli*-specific phages between goats and beef cattle ($p = 0.03913$), and sheep and beef cattle ($p = 0.006884$). This could be attributed to the null isolation of *E. coli*-specific phages in beef cattle. Conversely, Fisher's exact test did not observe significance in *P. aeruginosa*-specific phages between goats and beef cattle ($p = 0.09565$), and sheep and beef cattle ($p = 1.000$) (Table 5). This study had expected significance in the latter two comparisons given the null isolation of *P. aeruginosa*-specific phages in beef cattle. The lack

of detectable significance might be attributable to the low expected probability of isolating *P. aeruginosa*-specific phages (P = 0.100).

When Fisher's exact test was used to test for independence in phage isolation rates within each livestock type, significance was noted comparing *E. coli* and *S. aureus*-specific phages to *P. aeruginosa*-specific phages in sheep ($p = 0.03518$). This can be attributed to the low isolation rates of *P. aeruginosa*-specific phages compared to high isolation rates of *E. coli* and *S. aureus*-specific phages. Significant difference was also noted when comparing both *E. coli* and *P. aeruginosa*-specific phages to *S. aureus*-specific phages in beef cattle ($p = 0.001648$) (Table 5). This was most likely due to the fact only *S. aureus*-specific phages were isolated from beef cattle.

TABLE 7. Statistical analysis of Fisher's exact test. Statistical *p*-values left of the grey divider are results of paired comparisons of phages between livestock types. Statistical *p*-values to the right of the grey divider are results of paired comparisons of phages within each livestock types.

		E. coli			***S. aureus***			***P. aeruginosa***		
Phage specific to		***Goats***	***Sheep***	***Cattle***	***Goats***	***Sheep***	***Cattle***	***Goats***	***Sheep***	***Cattle***
E. coli	***Goats***				1.000			1.000		
	Sheep	0.6960				1.000			0.03518	
	Cattle	0.03913	0.006884				0.001648			1.000
S. aureus	***Goats***							1.000		
	Sheep				0.4244				0.03518	
	Cattle				0.1107	0.4757				0.001648
P. aeruginosa	***Goats***									
	Sheep							0.2940		
	Cattle							0.09565	1.000	

Visual observation of an X-Y plot of bacteriophage isolation successes factored against livestock type and bacteriophage type suggests that phages specific to *S. aureus* were the most prevalent across all three livestock (Fig. 1).

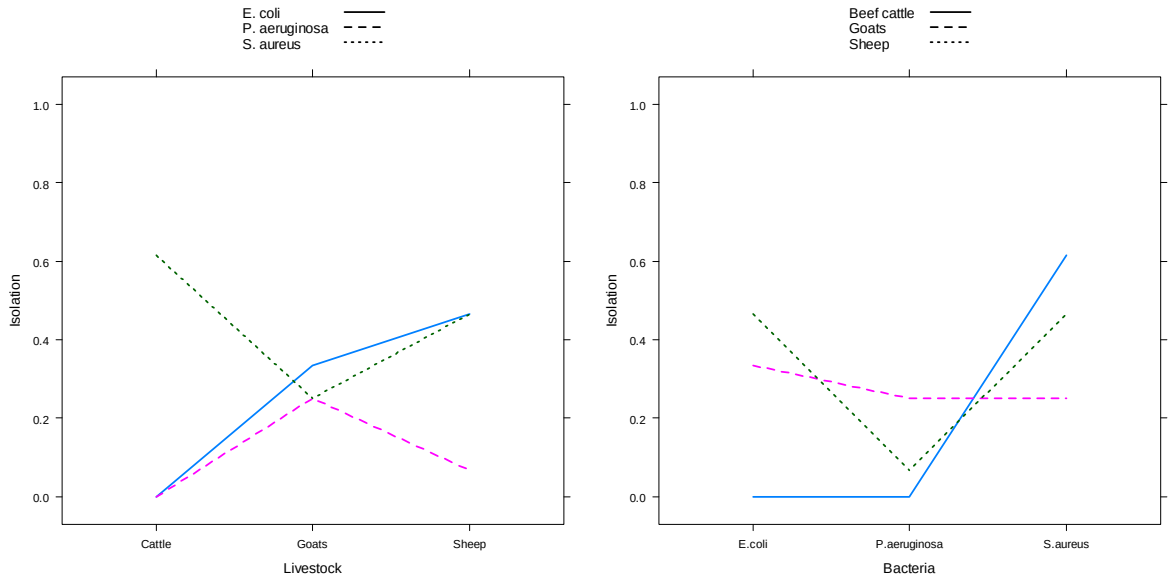

FIG. 1. X-Y plot of bacteriophage isolation against livestock type and bacteriophage type. Livestock type and bacteriophage type are both utilized as grouping (left and right respectively) for comparative purposes.

Analysis of bacteriophage isolation by the nested set modeling indicated that both livestock type and the bacteria-specific phage type effectively influenced isolation success, with an existing interaction between both explanatory variables (AIC = 116.0). This was also reinforced by visual observation of an x-y plot comparing both bacteriophage type and livestock type against isolation success (Fig. 1).

Visual observation of an X-Y plot of bacterial isolation successes against sampling type and livestock type showed that there was minimal difference in the isolation of *S. aureus* and *P. aeruginosa* (Fig. 2; top-left and top-right). It also appeared that the overall success rate of bacteria isolation rose when freshly-collected homogenate were utilized when compared against retentate (Fig. 2; top-left and bottom-left).

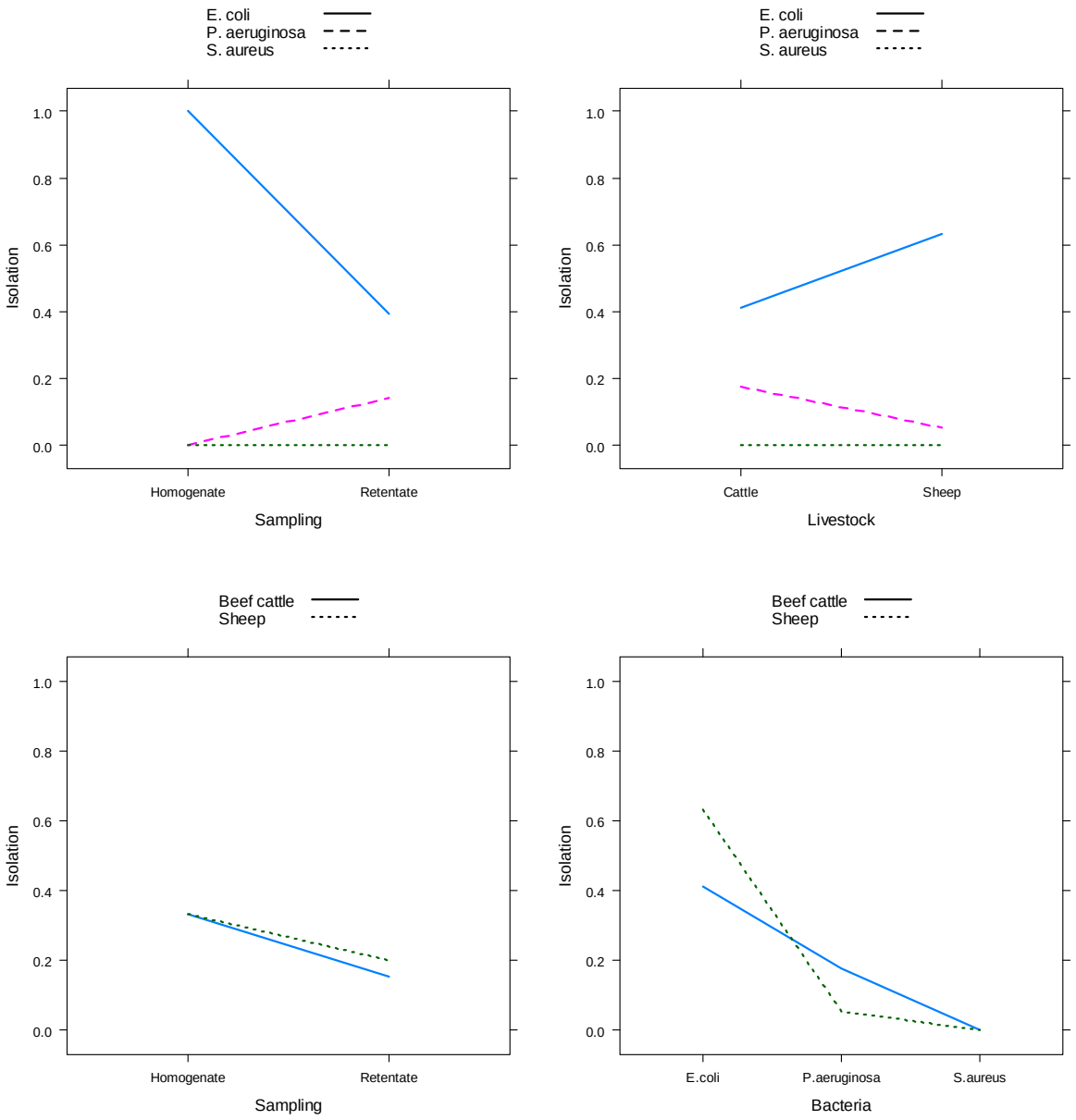

FIG. 2. X-Y plot of bacteria isolation against bacteria species, livestock type and sampling method. Bacteria isolation was compared against sampling method (top-left) and livestock type (top-right), with bacteria species as grouping. Bacteria isolation was also compared against sampling method (bottom-left) and bacteria species (bottom-right), with livestock type as grouping.

Analysis of the interactions between variables in bacteria isolation via nested set model reveals that isolation success was mostly affected by bacterial species and sampling type with interaction existing between both explanatory variables (AIC = 74.49). The chi-squared *p*-

value was calculated at $< 2.200 \times 10^{-16}$. Visually, this could be confirmed by the fact that the largest spatial differences in the trendline can be seen in the x-y plot comparing bacteria isolation against bacteria species and sampling method (Fig. 2; top-left). Livestock type did appear to also have a significant correlation with bacteria species-dependent isolation (AIC = 85.72), with a *p*-value of 7.740×10^{-09} (Fig. 2; top-right). In both comparisons, an interaction existed between bacterial species and sampling method, and between bacterial species and livestock type. Finally, when observing the explanatory power of both livestock type and sampling method with interaction between the explanatory variables, Chi-squared analysis resulted in an AIC of 82.17 and a *p*-value of 0.6334. This suggested that livestock type and sampling method influence bacteria isolation independent of each other.

DISCUSSION

This study successfully isolated bacteriophages specific to *E. coli*, *S. aureus* and *P. aeruginosa* from goats and sheep. Only *S. aureus*-specific phages were isolated from beef cattle. This thesis also demonstrates the feasibility of isolating phages specific to *E. coli* and *S. aureus* from sheep and goats using fecal samples. Furthermore, this study reports the first attempt to successfully isolate *P. aeruginosa*-specific phages from drinking water taken from water troughs. Likewise, this is the first reported isolation of *S. aureus*-specific phages from beef cattle, sheep and goats, via fecal samples, and *P. aeruginosa*-specific phages from goats, via fecal and drinking water samples.

This study notes the absence of *P. aeruginosa*-specific phages from beef cattle. No previous research has reported successful phage isolation prior to this study in contrast to positive results reported in sheep and goats. This might suggest that the intestinal tracts of beef cattle are not conducive to *P. aeruginosa*-specific phages. Alternatively, the isolation of *E. coli*-specific phages, which should be expected, was unsuccessful in contrast to positive reports from previous studies (Niu et al. 2007, Oot et al. 2009).

The successful isolation of *E. coli*-specific phages in 50% of the water troughs utilized by beef cattle conflicts with the null isolation in fecal samples, despite the greater sample size of the latter. However, the isolation of *E. coli*-specific phages in one of unused water troughs tested might suggest that the bacteriophage isolated in beef cattle-derived water samples may not actually have originated from the livestock. The *E. coli*-specific phages in unused water troughs could have originated from contact with a previous animal. This also suggests a substantial ability of the bacteriophage to persist in water environments, possibly replicating off a resident host bacteria population.

Comparison between phage quantification of water and fecal samples suggested that the latter tended to have a higher probability from which to successfully isolate phages. This was to be expected as livestock may have shed phages directly with the feces during defecation. *E. coli* showed the highest prevalence levels among all bacterial species in both fresh homogenate and filter retentate samples, with isolation results of 100% and 39.3% respectively. Only *E. coli*-specific phages appear to be naturally abundant enough to achieve phage titration, even though this distribution may be uneven. *S. aureus* and *P. aeruginosa*-specific phages, even if present, appear to be present in very low levels. This is expected as *E. coli* can be naturally found in the guts of ruminants, but perhaps not the other two species (Oot et al. 2007). The differences in *E. coli* isolation rate between sampling types may be attributable to the loss of viability due to the amount of time that filters were kept in storage.

S. aureus is the only bacterium tested that does not naturally colonize gastrointestinal tracts. *S. aureus* bacteria, despite the high isolation rate of 45.0% of its associated bacteriophage, were not successfully isolated from any of the samples. It is quite possible shedding of *S. aureus*-specific bacteriophages from the skin has contributed to phage presence in fecal samples. It was also observed that all livestock often trampled on their own feces, increasing the opportunities of transmission of phage to the fecal sample. However, it should not be assumed that only bacteriophages were transferred to the fecal samples. It is possible that *S. aureus* bacteria were transferred as well but did not survive. The ability of *S. aureus* to persist in an open air environment is not known, but it should be noted that aeration can reduce the persistency of other bacterial species such as *E. coli* (Kudva et al. 1998). Alternatively, *S. aureus* phages may have colonized the gastrointestinal tract of the livestock. The various livestock had been observed to lick their own and each other's skin which may

have led to the unintentional ingestion of *S. aureus* bacteria and associated phages. It is also possible that the *S. aureus* bacteria were unable to survive the internal environmental conditions of the gastrointestinal tract, but the phages have successfully persisted and maintained their populations by replicating off new ingestions of bacteria. *E. coli* phages have been reported in gastrointestinal tracts, and deliberately increasing the host bacteria population has reportedly increased phage counts, lending support to this alternate explanation (Oot et al. 2007).

Examination of the Fisher's exact tests on isolation rates of bacteriophage isolation revealed no significant difference when comparing the absence of bacteriophages specific to *P. aeruginosa* in beef cattle to successful isolation in sheep and goats. This may be related to the low numbers of *P. aeruginosa*-specific phages expected overall. It is possible that increasing the sample size could eventually result in positive isolation of *P. aeruginosa* phages in cattle. Thus, this study could not rule out that *P. aeruginosa*-specific phages were not present in cattle.

Phages specific to *E. coli*, *S. aureus* and *P. aeruginosa* were all isolated in sheep and goats, but only *S. aureus*-specific phages were isolated in beef cattle fecal samples. Analysis via Fisher's exact tests observed significant differences in *E. coli*-specific phages in beef cattle. However, no significant differences were observed when comparing *P. aeruginosa*-specific phage isolation rates between livestock, although the isolation rates of *P. aeruginosa*-specific phages between goats and beef cattle were very close to significance ($p = 0.09565$).

Overall, statistical analysis using Fisher's exact test appeared to suggest that bacteriophage isolation was influenced by the type of livestock. Continuation of the analysis

via nested set modeling has indeed shown that a significant correlation existed between livestock type and isolation rates of the different bacteriophages. This study suggests the possibility that a combination of spatial isolation and opportunistic cross-species infection may explain this observation. Alternatively, bacteriophages have been shown to have a higher persistency than host bacteria, and it could be possible that *S. aureus* counts may have deteriorated to a low enough level that isolation was not possible even with enrichment while phages still remained viable (Kudva et al. 1998). Differences in phage and bacteria persistency will be discussed later in the discussion.

This research noted that cattle were spatially isolated from sheep and goats, being either on grazing pastures or housed in holding stalls distanced from the latter two livestock. This spatial isolation may explain the differences in phage isolation between cattle and the other two livestock. In contrast, sheep and goats were housed in adjacent holding stalls. The close proximity between sheep and goats and the similarity in phage presences in the two livestock suggest that cross-species transmission had occurred. Perhaps the single successful isolation of *P. aeruginosa*-specific phages in sheep may have originated from a cross-species infection from goats possibly via sharing of water sources or feed. If cross-species infection occurred, then phage isolation probabilities between livestock types are not independent. Likewise, this could also explain the absence of *E. coli* and *P. aeruginosa*–specific phages in beef cattle, in spite of the successful isolation of the respective host bacteria. The spatial isolation of beef cattle from the other livestock may have prevented cross-species colonization of the two bacteriophages. This raises the possibility that bacteriophage distributions in livestock may be decided mainly by infection and opportunities for phage shedding and cross-species transmission.

The negative correlations observed in the results are support by previous studies by Niu et al. (2009) and Oot et al. (2007). However, this is in conflict with the positive correlations between phages and host bacteria as reported by Contreras-Coll et al. (2002), and Kenard and Valentine (1974). The studies reporting negative correlations carried out bacteria and phage isolation on cattle-derived fecal material, paralleling the methodologies of this thesis (Niu et al. 2009, Oot et al. 2007). In contrast, the studies reporting positive correlation were focused on samples from large water bodies (Contreras-Coll et al. 2002, Kenard and Valentine 1974). A possible explanation is that an expansive water environment has the effect of diluting viral abundance, thus decreasing the rate of encounter between bacteriophages and potential host (Weinbauer 2004). This in turn reduced the rate of infection and lysis of the bacteria. In contrast, fecal material may simply be more compact, leading to a higher encounter rate between phages and host bacteria compared to water sources. A high encounter rate would then result in a phage-induced reduction in natural host bacteria populations (Garcia et al. 2009, Niu et al. 2009, Oot et al. 2007). Finally. Oot et al. (2007) also suggested that bacteria colonization of a region can also be hindered by the presence of an already-existing resident phage population.

The scope of this study only encompasses lytic phages and no measures were taken to account for temperate phages. Compared to lytic phages, temperate phages are capable of entering a lysogenic life cycle where the viral genome is remains within the infected host as a prophage and achieving a state of dormancy (Gill and Hyman 2010, Weinbauer 2004). Presence of temperate bacteriophages is expected to result in fewer reported phage isolations than the true isolatable rate. To include temperate phages in a study, phage isolation would require exposure to UV light, mitomycin C or chloroform (Garcia et al. 2009, Sechaud and

Kellenberger 1956, Weinbauer 2004). Such treatments would lead to damage to host DNA, which is the most common the trigger to induce lysis in infected bacteria (Gill and Hyman 2010). It is conceivable that the levels of temperate phages may outnumber the lytic phages and consequently be responsible for the low bacteria prevalence despite not being represented in phage isolation results.

Bacteriophages, even when specific to the same host bacteria, may be different in their virulence. In 1999, Kudva et al. (1999) reported that different *E. coli* O157:H7-specific phages differ in their efficiency in reducing the titer count of bacteria populations. However, in cultures involving just one type of bacteriophage, phage-resistance developed among the *E. coli* bacteria within five days (Kudva et al. 1999). When bacteria cultures are simultaneously infected with three different *E. coli*-specific phages, complete bacterial death was observed in some cases. Even when infected with mixed phage populations, an incubating temperature of 37°C and aeration showed to be significant deciding factors in complete removal of bacterial culture.

Oot et al. (2007) suggested a negative correlation in their study, being able to isolate *E. coli*-specific bacteriophages in ~97% of fecal samples but the bacteria were only isolated in ~27% of the same samples. Thus, the idea that ubiquitous phage populations may reduce or even remove host bacteria populations from natural environments is not new (Niu et al. 2009, Oot et al. 2007). According to a study by Kudva (1998), phage presence, combined with aeration and appropriate temperatures, can completely remove a bacterial population of *E. coli* in vitro. However, this required a high temperature of 37°C which was not found in the environment from where the fecal samples were collected. Additionally, for the bacteria

cultures studied by Kudva (1998) to be completely removed, they had to be simultaneously inoculated with multiple different phage strains.

The time interval that bacteria in fecal material were exposed until collection may have also influenced isolation success of host bacteria. While efforts were made to ensure collected fecal samples were as recently vacated as possible, it is acknowledged that livestock feces were subject to open environmental conditions for an undeterminable amount of time which could reduce bacteria population. According to a study by Kudva et al. (1998), artificial aeration of fecal material severely decreased the survival span of *E. coli* from one year to just four months in sheep feces, and to only 47 days in bovine feces.

The low association between isolation of the bacteriophages and their host bacteria may also be attributable to the differences in persistency. Bacteriophage isolation was often carried out within 24 hours of sample collection, and bacteria isolation began only after all phage isolation was complete. The filters were often stored in a refrigerated room for up to three months before the retentate could be tested for the presence of host bacteria. This could lead to a systematic error arising from timing discrepancies as bacteria within water and fecal samples may have experienced significant loss of viability during storage and even before initial collection. Bacteria isolation from homogenate used in the processing of freshly collected fecal samples was intended to compensate for this error by attempting bacteria isolation within 24 h of collection and the results used for comparison purposes.

Oot et al. (2007) emphasized the ubiquitous nature of phage populations, although bacteriophage counts can only rise after an increase in host bacteria population. Hence, the successful isolation of phages in a larger quantity of samples than those resulting in positive

bacteria isolation should not be unexpected. It is quite possible that bacteriophages have a greater persistence rate than their host bacteria.

While comparison of bacteria and bacteriophage isolation of *E. coli* supported the notion that the loss of bacteria viability occurred during storage, the results of *P. aeruginosa* seem to indicate bacteria growth had occurred while in storage. *P. aeruginosa* could only be isolated from filter retentate (14.3%), and not from homogenate. All three bacteria are capable of growth in anaerobic environments, which rules out the possible explanation that *P. aeruginosa* could simply grow in the anaerobic conditions of the filters while *E. coli* and *S. aureus* could not (Belay and Rasooly 2002, Tran and Unden 1998, Yoon et al. 2002). A second possibility is that bacterial growth of *P. aeruginosa* may be due to its nutritional versatility. Generally, a simple medium containing mineral salts and glucose is sufficient to sustain prototrophic *P. aeruginosa* (Barth and Pitt 1995). In comparison, *S. aureus* requires the presence of amino acids for bacterial growth (Gladstone 1937). For example, *S. aureus* strain S-6 requires 11 amino acids (glycine, valine, leucine, threonine, phenylalanine, tyrosine, cysteine, methionine, proline, arginine, and histidine), and three vitamins (thiamine, nicotinic acid, and biotin), in addition to inorganic salts (Mah et al. 1967). However, *E. coli* similarly needed only mineral salts and glucose as a carbon source (Billen and Lichstein 1950). Thus, the second explanation is shown to be unlikely as well. This study thus suggests that the one successful isolation of *P. aeruginosa* in the retentate compared to the null isolation in homogenate may be a matter of coincidence.

This thesis investigated possible antibiotic usage which might have affected bacteria isolation in livestock. According to personal correspondence with Dr. Cody Scott, Chlortetracycline was administered to lamb calves only. Chlortetracycline is a broad

spectrum antibiotic effective against intestinal bacteria (Di Stefano et al. 2000). As all sheep fecal samples were sourced from adults, it is unlikely that the antibiotic use affected bacteria isolation, as bacterial population recovery was highly likely to recover by the time of sampling. Thus, the hypothesis that antibiotics in feed affected the results of bacteria isolation can be rejected.

Finally, this study also examined the possibility that different strains of bacteriophages may have been isolated even if they infect the same bacterial species. Different phage strains may have different specificity to different host bacteria strains as well (Garcia et al. 2007, Garcia et al. 2009). It is important to note that only one strain of each host bacterial species was used in this study. Some bacteriophages may not have been specific for that particular host. It is possible that more phages existed that were not isolated due to their specificity to bacterial strains different from those used in phage enrichment and isolation. An approach to address this is to utilize as many different strains of host bacteria that are available and reattempt phage isolation using the different bacteria strains. Bacteriophages from successful plaque isolation may also be further differentiated by observing for their ability to lyse different strains of host bacteria. Viral plaques from plaque assays may be extracted via autoclaved toothpicks and enriched with different host bacteria strains. Plaque assays or titrations may be used to observe if phage replication has occurred. An alternate approach is to utilize random amplification of polymorphism DNA (RAPD) to characterize the bacteriophages. This latter methodology was employed by Garcia et al. to differentiate *S. aureus*-specific phages (2009). An understanding of the strains of bacteriophages that were isolated may provide further information on the idea of opportunistic transmissions by observing the spatial patterns and distribution of phage strains among the different livestock.

Phage typing using bacteriophage strains isolated in this study might have aided in characterizing isolated bacteria into discernible strains (Garcia et al. 2009).

To summarize, possible future investigations could be carried out on the following issues: phage virulence, bacteria persistency, isolation of temperate phages, and characterization of phage and bacteria strains. Phage and bacteria titrations could be carried out to observe changes in population sizes of mixed cultures in vitro over time. The reduction in bacteria populations can serve as a quantifiable measure of the virulence of the phage. Similarly, bacteria persistency can be examined by quantification of a bacteria population taken from fecal samples with known exposure time to the open environment. It may also be advisable to observe for phage persistency using phage titrations as well to observe if phages tend to have a greater survival span than their host bacteria. This could explain the greater number of samples with isolated bacteriophages than the bacteria.

Future phage isolations can utilize UV light, mitomycin C or chloroform to induce lysis of lysogenized phages in enrichment cultures (Garcia et al. 2009, Sechaud and Kellenberger 1956, Weinbauer 2004). Phage isolation may also be reattempted using multiple different bacterial strains instead of only one strain per species to maximize bacteriophage isolation. Collected phages may be examined for their specificity to different bacterial strains. This allows phages to be characterized by strain and distribution of the same or different phage strains across livestock types may provide evidence to the possible hypothesis that spatial isolation between livestock determines phage presence.

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APPENDIX

Ingredients for Phage Agar

Nutrient broth (Acumedia)	4.8 g
NaCl (Mallinckrodt, Hazelwood, Mo.)	3 g
Dextrose/Glucose (Sigma, St. Louis, Mo.)	0.6 g
Agar, Bacteriological (Acumedia)	9 g
Distilled H_2O	600 ml

Autoclave
Time: 20 min
Temperature 121°C
Pressure: 16 PSI

Ingredients for Soft Phage Agar

Nutrient broth (Acumedia)	8 g
NaCl (Mallinckrodt, Hazelwood, Mo.)	5 g
Dextrose/Glucose (Sigma, St. Louis, Mo.)	1 g
Agar, Bacteriological (Acumedia)	8 g
Distilled H_2O	1000 ml

Autoclave
Time: 20 min
Temperature 121°C
Pressure: 16 PSI

Ingredients for Tryptic Soy Agar (TSA)

Trypticase Soy Agar powder (Acumedia)	40 g
Distilled H_2O	1000 ml
OR	
Tryptone (Difco)	17 g
Soytone (Difco)	3 g
Dextrose/Glucose (Sigma, St. Louis, Mo.)	2.5 g
NaCl (Mallinckrodt)	5 g
Dipotassium phosphate (K_2HPO_4)	2.5 g
Agar, Bacteriological (Acumedia)	15 g
Distilled H_2O	1000 ml

Autoclave
Time: 20 min
Temperature 121°C
Pressure: 16 PSI

Ingredients for Tryptic Soy Broth (TSB) with 7.5% NaCl

Tryptone (Difco)	17 g
Soytone (Difco)	3 g
Dextrose/Glucose (Sigma, St. Louis, Mo.)	2.5 g
NaCl (Mallinckrodt)	75 g
Dipotassium phosphate (K_2HPO_4)	2.5 g
Distilled H_2O	1000 ml

Autoclave
Time: 20 min
Temperature 121°C
Pressure: 16 PSI

Ingredients for Tryptone Broth (1%)

Tryptophan (Difco)	10 g
Distilled H_2O	1000 ml

Autoclave
Time: 20 min
Temperature 121°C
Pressure: 16 PSI

Ingredients for Asparagine Broth

Asparagine, DL (Sigma)	3 g
Anhydrous dipotassium hydrogen phosphate (K_2HPO_4)	1 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5 g
Distilled H_2O	1000 ml

Autoclave
Time: 20 min
Temperature 121°C
Pressure: 16 PSI